PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/00, G01N 33/53, A61K 35/16 — A1

(11) International Publication Number: WO 99/20787

(43) International Publication Date: 29 April 1999 (29.04.99)

(21) International Application Number: PCT/US98/21697

(22) International Filing Date: 15 October 1998 (15.10.98)

(30) Priority Data:
60/062,519 17 October 1997 (17.10.97) US

(71) Applicants *(for all designated States except US)*: THE SCHEPENS EYE RESEARCH INSTITUTE, INC. [US/US]; 20 Staniford Street, Boston, MA 02114 (US). BOSTON BIOMEDICAL RESEARCH INSTITUTE [US/US]; 20 Staniford Street, Boston, MA 02114 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: STREILEIN, J., Wayne [US/US]; 44 Neptune Street, Beverly, MA 01915 (US). XIE, Yong [CN/US]; Apartment #18J, 402 Rindge Avenue, Cambridge, MA 02140 (US). WANG, C.-L., Albert [US/US]; 29 Battle Green Road, Lexington, MA 02173 (US). LI, Yanhua [CN/US]; Apartment #13L, 402 Ridge Avenue, Cambridge, MA 02140 (US).

(74) Agents: HEINE, Holliday, C. et al.; Weingarten, Schurgin, Gagnebin & Hayes LLP, Ten Post Office Square, Boston, MA 02109 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: CONTROL OF APC-DEPENDENT T CELL ACTIVATION BY THE ALPHA-1 CHAIN OF HAPTOGLOBIN

(57) Abstract

A polypeptide present in normal human blood that interferes with the capacity of antigen presenting cells of the skin (Langerhans cells) to activate naive autologous T cells has been discovered and identified as the alpha-1 chain of the molecule haptoglobin. The haptoglobin alpha-1 chain is a selective inhibitor that acts only on antigen presenting cells and not on other cells of the immune system such as T lymphocytes. Therapeutic compositions containing human haptoglobin or its alpha-1 chain may be administered to a patient to interfere with Langerhans cells/dendritic cell-dependent activation of T cells and, therefore, to suppress T-cell dependent inflammation *in vivo*. Specific treatable inflammatory skin diseases include psoriasis, mycosis fungoidosis, atopic dermatitis, exfoliative dermatitis and Sezary's Syndrome. Other autoimmune, T cell dependent inflammations and infectious diseases would be treatable by the methods of the invention including prevention of transplant rejection (e.g. Graft Versus Host Disease), vaccine development, and therapy for infectious diseases where antigen presenting cells play a central role in the pathogenesis.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TITLE OF THE INVENTION

CONTROL OF APC-DEPENDENT T CELL ACTIVATION BY THE ALPHA-1 CHAIN OF HAPTOGLOBIN

CROSS APPLICATION TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application No. 60/062,519, filed October 17, 1997, the whole of which is hereby incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The research leading to this invention was supported in part by United States Government funds under Grant No. AR44130 from the National Institutes of Health. Therefore, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Immune protection of the skin against invading pathogens is initiated by antigen presenting cells located within the skin itself. Epidermal Langerhans cells (LC) are strategically located within the epidermis for this role, and they express cell surface molecules appropriate for presentation of antigens to T cells. When placed *in vitro*, freshly obtained LC have been demonstrated to process and present a wide variety of antigens to primed and alloreactive T cells. Moreover, these dendritic bone marrow-derived cells have been implicated *in vivo* as crucial antigen presenting cells in the induction of contact hypersensitivity to epicutaneously applied haptens.

However, when cultured *in vitro* for 2-3 days in the presence of GM-CSF or keratinocytes, Langerhans cells acquire a unique functional ability to activate autologous naive T cells, even in the absence of antigens (Schuler et al., J. Exp. Med. 161:526, 1985; Xie et al., J. Dermatol. Sci. 12:263-274, 1996). Accumulating evidence indicates that

failure to prevent this functional transformation of Langerhans cells *in vivo* may induce rampant T cell proliferation and trigger skin diseases, such as psoriasis (Demidem et al., J. Invest. Derm. 97:454, 1991), as well as accelerate the dissemination of HIV to T cells (Soto-Ramirez et al., Science 271:1291, 1996; Ayehunie et al., AIDS Res. Hum. Retroviruses 11:877, 1995; Girolomoni et al., Immunol. 87:310, 1996). It has recently been reported that an unknown murine serum factor can species-specifically inhibit LC functional transformation *in vitro* and can maintain a homeostatic role *in vivo* (Xie et al., J. Dermatol. Sci. 12:263, 1996). Furthermore, human dendritic cells cultured in the presence of human serum failed to undergo the expected functional comparable transformation (O'Doherty et al., Immunol. 82:487-493, 1994).

Known agents, such as the corticosteroids and cyclosporin-A, that have the ability to inhibit antigen presentation are non-tissue specific and have effects and toxicities involving many cell types and tissues. Novel agents that could specifically inhibit antigen presentation would be highly desirable.

BRIEF SUMMARY OF THE INVENTION

We have identified a polypeptide present in normal human blood that interferes with the capacity of antigen presenting cells of the skin (Langerhans cells) to activate naive autologous T cells. This is the first example of a selective inhibitor that acts only on antigen presenting cells and not on other cells of the immune system such as T lymphocytes. This polypeptide has been identified as one of the chains of the molecule haptoglobin, specifically the alpha-1 chain.

Haptoglobin is a well known molecule whose best known biological function is the capture of cell free hemoglobin in order to prevent both iron loss and kidney damage during hemolysis. Haptoglobin is also a positive acute-phase

protein. The ability of the alpha-1 chain of haptoglobin to interfere with Langerhans cell function is unprecedented.

Thus, in one aspect, the invention is directed to pharmaceutical compositions comprising the alpha-1 chain of haptoglobin (or active portions thereof), or the entire haptoglobin molecule, and methods for their use to suppress T-cell dependent inflammation *in vivo* by inhibiting the ability of antigen presenting cells to activate T cells. For example, specific treatable inflammatory skin diseases include psoriasis, mycosis fungoidosis, atopic dermatitis, exfoliative dermatitis and Sezary's Syndrome. Additionally, a number of autoimmune, T cell dependent inflammations and infectious diseases would be treatable by the methods of the invention. Specific examples include prevention of transplantation rejection (e.g., Graft Versus Host Disease), vaccine development, and therapy for infectious diseases where antigen presenting cells play a central role in the pathogenesis. Furthermore, the methods of the invention can also be used to inhibit Langerhans cell susceptibility to infection with viruses, especially HIV.

In another aspect, the invention is directed to pharmaceutical compositions comprising an antagonist to human haptoglobin-haptoglobin receptor binding and to methods for their use in stimulating a T-cell dependent immune response *in vivo* by stimulating the ability of antigen presenting cells to activate T cells. This treatment would be appropriate in circumstances in which antigen presenting cells, at the T cell activation site, were inappropriately flooded with haptoglobin.

BRIEF DESCRIPTION OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the effect of heat treatment on the abilities of human and mouse serum to prevent mouse epidermal LC cells from acquiring *in vitro* the capacity to activate naive, syngeneic T cells. BALB/c LC were cultured in medium with or without supplementation with 10% human serum, heated human serum, mouse serum and heated mouse serum, respectively. After 3 days, the LC were harvested and used as stimulators in syngeneic MELR. Six days later, T cell proliferation was assayed as described in Materials and Methods. (A) unstimulated T cells: (B) T cells stimulated with LC that had been cultured for 3 days in medium alone; or (C) in medium supplemented with human serum; or (D) in medium supplemented with heated human serum; or (E) supplemented with mouse serum; and (F) supplemented with heated mouse serum. (*) indicates mean values significantly lower than positive control (B), $P < 0.001$;

Fig. 2A shows the abilities of >100 kD and <100 kD fractions of heated human serum to prevent epidermal LC cells from acquiring *in vitro* the capacity to activate naive, syngeneic T cells. BALB/c epidermal LC were cultured in medium with or without supplementation with 10% unfractionated heated human serum, <100 kD fraction and >100 kD fraction of heated human serum, respectively. After 3 days, the LC were harvested and used as stimulators in syngeneic MELR. Six days later, T cell proliferation was assayed as described in Materials and Methods. (A) unstimulated T cells; (B) T cells stimulated with LC that had been cultured for 3 days in medium alone; or (C) in medium supplemented with heated human serum ; or (D) supplemented with <100 kD fraction of heated human serum; and (E) supplemented with >100 kD fraction of heated human serum.

(*) indicates mean values significantly lower than positive control (B), $P < 0.001$;

Fig. 2B shows SDS-PAGE analysis of (a) heated serum fraction that contains protein species greater than (lane 1) or less than (lane 2) 100 kDa separated by ultrafiltration with Centricon-100 tube (Amicon, Beverly, MA); (b) protein component of inhibitory fraction (#5 in Fig.2c) purified by HPLC (lane 3). Lane 4 is the molecular weight standard;

Fig. 2C is a profile of the less than 100 kDa fraction of heated human serum analyzed by reversed-phase HPLC. Eight major peaks appeared, and were collected in different fractions. Each fraction after lyophilization was dissolved in water and added to cultures of epidermal LC to test the inhibitory effect. After 3 days LC were used to stimulate syngeneic naive T cells. Among those fractions only fraction #5, eluted out between 40-43% acetonitrile gradient, consistently displayed the inhibitory effect (see Fig. 2d);

Fig. 2D shows the ability of fraction #5 (see Fig. 2c) to prevent LC from acquiring *in vitro* the capacity to activate naive, syngeneic T cells. BALB/c epidermal LC were cultured in medium supplemented (or not) with proteins lyophilized from fraction #5 in Fig. 2c and a mixture of proteins lyophilized from the rest of the fractions except for #5. After 3 days, the LC were washed and used as stimulators in syngeneic MELR. Six days later, T cell proliferation was assayed as described in Materials and Methods. (A) unstimulated T cells; (B) T cells stimulated with LC that had been cultured for 3 days in medium alone; or (C) in medium supplemented with proteins of fraction #5; or (D) supplemented with proteins of fractions other than fraction #5; and (E) supplemented with less than 100 kDa fraction of heated human serum. (*) indicates mean values significantly lower than positive control (B), $P < 0.001$;

Fig. 3 shows the ability of Hp and heated Hp to prevent mouse epidermal LC from acquiring *in vitro* the capacity to activate naive T cells, and the effect of heated Hp on T cell

proliferation. BALB/c epidermal LC were cultured in medium with or without supplementation with 0.25 mg/ml purified Hp, heated Hp from pooled human serum (Sigma, St.Louis, MO). After 3 days, the LC were washed and used as stimulators in syngeneic MELR. Six days later, T cell proliferation was assayed as described in Materials and Methods. (A) unstimulated T cells; or (B) T cells stimulated with LC that had been cultured for 3 days in medium alone; or (C) in medium supplemented with Hp; (D) supplemented with heated Hp; and (E) T cells, in the presence of heated Hp, stimulated with LC that had been cultured for 3 days in medium alone. (*) indicates mean values significantly lower than positive control (B), $P < 0.001$;

Fig. 4 shows FACS analysis of Ia and B7-2 expression on freshly obtained mouse LC, and LC cultured in medium supplemented (or not) with heated Hp. LC were fixed with 5% formaline in PBS and stained with FITC-conjugated mAb anti-mouse I-Ad (Pharmingen, San Diego, CA) followed by PE-conjugated anti-mouse B7-2 (Pharmingen). FITC-mouse IgG3 and PE-rat IgG2a (Pharmingen) were used as isotype controls. A: freshly obtained LC; B: LC cultured with medium alone, and C: LC cultured with medium supplemented with heated Hp;

Fig. 5 shows the effect of Hp-free serum on the ability of human epidermal LC to acquire *in vitro* the capacity to activate autologous, naive T cells. Human epidermal LC were cultured in medium supplemented with 20% Hp-free human serum, 20% Hp-free human serum+purified Hp (0.25 mg/ml), or 20% normal human serum. After 3 days, the LC were washed and used as stimulators in human autologous MELR. Six days later, T cell proliferation was assayed as described in Materials and Methods. (A) unstimulated T cells; (B) T cells stimulated with LC that had been cultured for 3 days with 20% Hp-free serum; or (C) with 20% Hp-free serum+purified Hp; or (D) with 20% normal human serum. (*) indicates mean values significantly higher than inhibitory control (D), $P < 0.01$. Hp-free human serum was obtained by passing serum through

immunoaffinity column of anti-human haptoglobin antibody-Sepharose. (Depletion of Hp from serum was confirmed by Western Blotting assay.);

Fig. 6 is a dose response curve of the inhibitory effect of Hp on LC functional transformation in culture. Human epidermal LC were cultured in medium supplemented with 0.1, 0.2 and 0.4 mg/ml Hp, respectively. After 3 days, the LC were washed and used as stimulators in human autologous MELR. Six days later, T cell proliferation was assayed as described in Materials and Methods. (A) unstimulated T cells; (B) T cells stimulated with LC that had been cultured in medium alone; or (C) T cells stimulated with LC cultured in medium supplemented with 0.4 mg/ml Hp; or (D) with 0.2 mg/ml Hp; or (E) with 0.1 mg/ml Hp. (*) indicates mean values significantly lower than control (A), $P < 0.01$;

Fig. 7 shows confocal laser scanning microscopy of LC in human epidermis stained with anti-HLA and anti-Hp antibodies. Human epidermis were stainded as described in the method and examined with a Leica TCS 4D confocal laser microscope. A dendritic epidermal cell indicated with positive staining of Hp (red) displayed positive staining of HLA (green) in freshly obtained epidermis. The co-localization of Hp and HLA can be viewed in HLA + Hp double staining; and

Fig. 8 shows confocal laser scanning microscopy of LC stained with anti-HLA (green) and anti-Hp (red) antibodies in freshly obtained epidermis (A), in epidermis cultured for 3 days in Hp-free medium (B), and in epidermis cultured for 3 days in Hp-containing medium (C).

DETAILED DESCRIPTION OF THE INVENTION

Epidermal Langerhans cells (LC), a subpopulation of dendritic cells, are the primary antigen presenting cells of the skin. As such, they have proven to be functionally plastic. Their precise antigen presenting function is dictated by soluble factors in the microenvironment in which

they reside. Epidermal LC carry out two distinct antigen presenting roles in cutaneous immunity. Within the epidermis of normal individuals, LC capture, process and present antigens in a manner that can directly activate previously primed, but not naive, T cells. These are also features of LC freshly prepared from skin and assayed *in vitro*. When LC are stimulated *in vitro* with GM-CSF and IL-1, their functional properties transform in a way that enables the cells to present antigens to naive, unprimed T cells. It is believed that a similar transformation occurs when LC migrate from the skin to draining lymph nodes during the induction of immunity to cutaneous antigens. The ability of cultured LC, but not fresh LC, to activate syngeneic or autologous T cells in the absence of cognate antigen is a convenient method to distinguish the two polar functional phenotypes of LC.

We have unexpectedly discovered, in normal human serum, a 9,000-10,000 molecular weight polypeptide that has the capacity to inhibit the *in vitro* transformations of LC functional properties. This polypeptide has been subjected to sequence analysis and has been identified as the alpha-1 chain of the serum protein haptoglobin.

Haptoglobin is a well known molecule whose existence and certain functions have been known for almost a hundred years. The best known biological function of haptoglobin is capture of hemoglobin to prevent both iron loss and kidney damage during hemolysis. Haptoglobin is also a positive acute-phase protein and is characterized by a molecular heterogeneity with three major phenotypes: Hp 1-1, Hp 2-2, and the heterozygous Hp 2-1. Although haptoglobin is found in serum of all mammals, this polymorphism exists only in humans. The geographic distribution of haptoglobin phenotypes has been under a strong genetic pressure, and functional differences have been described among the three phenotypes. Haptoglobin polymorphism appears to be related to immune response and to autoimmune and inflammatory disorders.

Haptoglobin consists of two different polypeptide chains, the α-chain and the β-chain. The β-chain (40 kD) is heavier than the α-chain and is identical in all haptoglobin types. Haptoglobin polymorphism arises from variant α-chains. The Hp 1-1 phenotype expresses only α_1-chains (8.9 kD). α_2-Chains (16 kD) are present in Hp 2-2 and Hp 2-1. The higher hemoglobin binding capacity of Hp 1-1 and the association of the Hp-2 allele with higher immune reactivity, appear to have contributed to the selection and worldwide distribution of haptoglobin alleles.

The haptoglobins display immunoregulative abilities, which can be immunosuppressive by inhibiting lymphocyte reactivity, or immunoinductive by influencing IgM biosynthesis adapted to the functional requirements. Hp 2-2 has a stronger effect than the other two haptoglobin phenotypes (Lange, V., Anthropol. Anz. 50: 281-302, 1992).

In clinical terms, Hp 2-2 is associated with higher 5-year mortality of individuals with HIV infection (40% vs. 20% in the Hp 1-1 and 2-1 phenotypes) and is also overrepresented in patients with autoimmune disease. (Langlois et al., Clin. Chem. 42: 1589-1600, 1996). These data were suggested as supporting the concept of Hp 2-2 (alpha-2 chain) being an immunomodulating molecule (Delanghe, J., et al., Proc. of the XVI Int'l Congr. of Clin. Chem., A144, July 8-12, 1996).

Isolation of an inhibitory factor for Langerhans cell transformation from human serum.

Our first attempts to isolate the LC inhibitory factor were hampered by data that were difficult to interpret. We found that when we cultured mouse LC with whole human serum and medium containing fetal calf serum (FCS), which is considered the best serum supplement for cell culture media, we could observe an inhibitory effect from human serum. However, when we used FCS-containing medium and partially purified human serum, we could not detect LC inhibitory activity. These data suggested that whole serum could

contain protective factors for the LC inhibitory factor, which were separated out in the partially purified fractions, thus exposing the partially purified LC inhibitory factor to interactions with some blocking agent.

After examining the crude purification protocol without success for modifications that could restore inhibitory activity within a partially purified fraction, we then focused on the culture conditions. We discovered eventually that the FCS in the culture medium was blocking the activity of the human LC inhibitory factor on mouse LC cell cultures. Through repeated experimentation, we discovered that partially purified human serum did display an inhibitory effect on mouse LC cells when cultured in the absence of FCS (i.e., serum-free medium). We then tried to develop a suitable culture medium without FCS by replacing it with other types of serum. Neither rabbit nor mouse serum was effective, as they interacted with and blocked the LC inhibitory factor. We eventually overcame the problem of finding suitable culture conditions by replacing FCS with human serum as a culture medium ingredient for murine LC.

The human factor was then isolated using a purification protocol consisting of serial procedures of heat treatment, ultrafiltration and high pressure liquid chromatography (HPLC). As the first step, human and mouse sera were heated to 85°C for 25 min to determine the heat stability of the factor(s). Heat-denatured proteins precipitated out of the sera following this treatment. The precipitates were removed by centrifugation, and the supernatants were added to culture wells containing fresh murine LC plus GM-CSF. This experiment was designed to determine whether the readily available murine LC could be used as an assay system during the purification procedure. As can be seen in Fig. 1, when the cultured LC were removed at 3 days and added to syngeneic, naive murine T cells, the latter cells proliferated poorly, indicating that the serum factor(s) was heat stable. Surprisingly, however, not only did the

supernatant of heat-treated mouse serum retain the capacity to prevent the functional transformation of mouse LC *in vitro*, but heat-treated human serum also inhibited murine LC from transforming *in vitro* in response to GM-CSF. This result was in contrast to observations on the effect of non-heat treated human serum. In non-heat treated human serum, the inhibitory factor was species specific. The ability of heated human serum to act on murine LC made it possible for us to carry out experiments to purify and identify the putative factor from human serum as the starting source and yet use the easily available murine LC as the assay system.

Given this determination, pooled human serum was then heat treated as described above. The clear supernatant was subjected to ultrafiltration (Centricon-100 filter) in order to separate it into fractions >100kD and <100 kD. An aliquot from each fraction was then added to culture wells containing freshly procured murine LC plus GM-CSF. After 3 days, the LC were removed and used to stimulate syngeneic murine T cells. As shown in Fig. 2A, only the fraction that contained molecules <100 kD displayed inhibitory activity. This result was also anomalous compared to the behavior of the factor in whole serum where the inhibitory activity was in the >100kD fraction.

Both the >100 kD and <100kD fractions were then analyzed on SDS-PAGE. As can be seen in Fig. 2B, a small number of unique bands were identified in the <100 kD fraction, indicating that candidate molecules exist in this fraction. The <100 kD fraction of heat-treated human serum was then subjected to reversed-phase HPLC. Fractions were eluted from a C8 analytic column with a 0 to 70% acetonitrile gradient (as described in Materials and Methods), and then tested individually for their ability to inhibit the functional transformation of murine LC *in vitro*. As shown in Fig. 2C, eight fractions were added individually to fresh murine LC and cultured for 3 days. Thereafter, the LC were removed and used to stimulate naive, syngeneic murine T cells. Only

fraction no. 5 displayed inhibitory activity (see Fig. 2D). The inhibition mediated by fraction no. 5 was somewhat less than that of the unfractionated <100 kD fraction of heated serum, probably due to losses during the HPLC and lyophilization purification procedures. Susceptability to pronase treatment verified that the inhibitory factor was proteinaceous.

As we had confirmed that the inhibitory factor was a protein, we next determined the purity of fraction no. 5 by SDS-PAGE; only a single band was identified. The mobility of the band on SDS-PAGE matched that of one of the unique bands present in the <100 kD fraction displayed in Fig. 2A.

Identification of the isolated serum inhibitory factor as the alpha-1 chain of haptoglobin.

Next, to identify the molecule in this band, the material was removed and subjected to amino acid sequencing. N-terminus sequencing of up to 20 residues was carried out on an automated gas phase sequenator (ABI 477A). The sequence was found to be:
ValAspSerGlyAsnAspValThrAspIleAlaAspAspGlyCysProLysProProGlu.
This sequence is identical to the N-terminal residues of the alpha-1 chain of human haptoglobin (Kurosky et al., Proc. Natl. Acad. Sci. USA 77:3388, 1980; Young et al., Proc. Natl. Acad. Sci. USA 80:5875, 1983). Mass spectrometry analysis revealed that the precise mass of the inhibitory molecule from heated human serum was 9,185 D. Since this it the actual size of the alpha-1 chain of Hp (14), we conclude that the inhibitory factor in human serum is either the alpha-1 chain of Hp, or the Hp molecule itself.

To support the above conclusion, purified human Hp was obtained from a commercial source (Sigma) and tested in our experimental system to confirm the inhibitory effect of this molecule on LC functional transformation. It has previously been shown that the concentration of haptoglobin is 0.4-3 mg/ml in normal human serum and 0.37 mg/ml in human cutaneous

interstitial fluid (Langlois et al., Clin Chem 42:1589, 1996; C. Clerc et al., Clin. Chim. Acta 189:181, 1990). We found that 0.25 mg/ml of human haptoglobin heated to 85°C for 25 min significantly inhibited murine LC from acquiring the capacity to activate syngeneic T cells (see Fig. 3). Unheated human Hp displayed no inhibitory activity. The inhibition of LC effected by heated Hp is not nonspecific, however, because direct addition of heated human Hp to syngeneic MELR failed to inhibit T cell proliferation (Fig. 3, bar E). The inhibition is also reversible, as cells incubated for 24 h in heated Hp followed by 48 h in Hp-free medium proved to be vigorous activators of syngeneic T cells. This result also suggests that Hp is not toxic to LC. The capacity of Hp to inhibit LC from acquiring the capacity to activate naive, syngeneic T cells may be related to effects on expression of MHC molecules and co-stimulatory molecules. To examine this possibility, murine LC were cultured with heated human Hp. Control LC were cultured in the absence of heated human haptoglobin. After three days, the cells were removed and analyzed by flow cytometry for expression of class II and B7-2 molecules. As shown in Fig. 4, LC cultured in the presence of heated human Hp expressed reduced class II molecules and reduced B7-2 compared to LC cultured in the absence of Hp.

Effects of human haptoglobin on functional transformation of human Langerhans cells *in vitro*

Having demonstrated that heat-treated human Hp inhibited the functional transformation of murine LC *in vitro* in a manner similar to heat-treated human serum, we next inquired whether Hp is the factor in human serum that inhibits human LC from acquiring the capacity to activate autologous naive T cells in culture. Human serum was passed through an anti-Hp antibody coupled immunoaffinity column in order to deplete the serum of Hp (deletion of Hp was confirmed by Western blot analysis). Hp-free serum was then added to

medium in which fresh human LC were cultured. Three days later, the LC were removed and used to stimulate autologous T cells. The results presented in Fig. 5 show that LC cultured in the presence of Hp-free serum readily activated autologous T cells. However, LC cultured in native human serum, and LC cultured in Hp-free serum to which exogenous Hp had been added, were poorly able to activate autologous naive T cells. A dose response experiment was then performed to determine the threshold concentration of Hp required for inhibitory activity. The data presented in Fig. 6 indicate that Hp concentration in the range of 0.1 mg/ml is capable of having a significant inhibitory effect on cultured LC. These findings provide strong evidence in support of the view that Hp in human serum is an inhibitory factor that prevents fresh LC from undergoing functional transformation *in vitro*.

In vivo and _in vitro_ evidence that serum haptoglobin is accessible to epidermal LC

To determine whether Hp is accessible to epidermal LC *in vivo*, anti-human Hp monoclonal antibody was used to stain freshly procured human epidermal sheets. Numerous epidermal cells with dendritic shape were observed with intense positive staining of Hp. When counter-stained with anti-HLA class II antibodies (which stains only LC in normal epidermis), all Hp-positive cells were confirmed to be LC (see Figs. 7 and 8). High power and confocal examination of these specimens suggested that the Hp was located primarily within cytoplasmic vesicles of LC (Fig. 7). However, when the same technique was used to examine epidermal sheets cultured for 3 days in Hp free medium, only sporadic Hp staining was found in the background, and almost all LC had lost their Hp staining (Fig. 8). Interestingly, when epidermal sheets cultured for 3 days in medium containing exogenous Hp or human serum were examined, many LC in the cultured sheets still displayed strong Hp staining (Fig. 8). Not all LC in these cultured sheets were equally stained;

some LC (about 50%) displayed only weak Hp staining. These data suggest that (a) Hp in LC is not synthesized by LC themselves; (b) serum Hp can gain access to epidermal LC *in situ*; and (c) LC may be able to endocytose and exocytose Hp. A similar result has been reported to the effect that endocytosed Hp in granuocytes can be actively exocytosed during phagocytosis of pathogens (Wagner et al., J. Immunol. 156:1989, 1996). These findings suggest the possibility that uptake of antigen by LC may simultaneously lead to exocytosis of Hp, and consequently, the loss of Hp may trigger the transformation of the LC into the mature functional phenotype.

Materials and Methods

Mice. Female BALB/c and C57BL/6 mice were obtained from The Jackson Laboratories (Bar Harbor, ME).

Cell culture medium. RPMI-1640 (Gibco, Grand Island, NY), supplemented with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM HEPES buffer saline, and 1% penicillin-streptomycin-fungizone (Gibco) was used as the primary culture medium. In mixed epidermal cell-lymphocyte reactions, 1 μg/ml indomethacin and 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO) were added. In cultures of mouse LC, 10% human serum (AB type, Hyclone, Logan, UT) was added. In cultures of human LC 10% FCS was added.

Preparation of epidermal LC. For human LC: skin blisters were raised on healthy volunteers after a Dermovac (Turko, Finland) instrument was placed on abdominal skin with negative pressure (about 250 mm Hg) for 2-3 h. The domes of skin blisters (epidermal sheet) were removed using sterile surgical procedures, and floated on cultured medium for 2-3 day culture. Cultured epidermal sheets were digested with 0.25% trypsin at 37°C for 15 min, disaggregated using a 5 ml syringe, and filtered through a 70 μm cell strainer (Falcon, Franklin Lakes, NJ). Recovered cells were used as cultured

LC. For mouse LC: shaved mouse skin was removed; subcutaneous tissues was scraped off and the skin sheet was floated on 0.25% trypsin-PBS for 1 h at 37°C. The epidermis was then separated with a fine forceps and incubated in 0.25 trypsin for an additional 10 min. The epidermis was further disaggregated using 10 ml syringe, filtered through 70 μm cell strainer, and the cell suspension used as LC.

Preparation of highly purified T lymphocytes. Axillary, inguinal and mesenteric lymph nodes were removed from naive mice, minced and resuspended in 2 ml T cell-enrichment buffer (R&D Systems, Minneapolis, MN). The cell suspension was loaded on mouse T cell-enrichment columns (R&D Systems), incubated at room temperature for 10 min, and then eluted with washing buffer. The first 6 ml of eluted cells were collected and referred to as purified naive T cells. More than 95% of these recovered cells were CD3 positive on the basis of flow cytometry analysis. Human T lymphocytes were purified in the same way from peripheral blood.

Immunofluorescence assays. Staining of human epidermal sheets: human epidermal sheets were fixed in acetone for 10 min, incubated with mouse anti-human haptoglobin monoclonal antibody (IgG1, 1:80, Sigma) for 60 min at 37°C and rinsed. Cy3-conjugated goat anti-mouse IgG1 immunoglobin (1:80, Caltag Laboratories, Burlingame, CA) was applied for 60 min in order to label haptoglobin. FITC-conjugated mouse anti-human HLA-DR,DP,DQ (IgG2a, 1:20, Pharmingen, San Diego, CA) was applied for 60 min to counter-stain for HLA. Controls consisted of substitution of primary anti-human haptoglobin antibodies with isotype mouse IgG1 (Caltag), and FITC-conjugated isotype mouse IgG2a (Pharmingen). FACS analysis of Ia, and B7-2 expression on mouse LC: freshly obtained, as well as cultured LC, were stained with FITC-conjugated mAb anti-mouse I-Ad (Pharmingen) followed by PE-conjugated anti-mouse B7-2 (Pharmingen). FITC-mouse IgG3 and PE-rat IgG2a (Pharmingen) were used as isotype controls.

Cells were analyzed on a COULTER EPICS XL flow cytometer using Workstation software Version 1.5 (Coulter, FL).

Mixed epidermal cell lymphocyte reactions (MELR). Purified naive lymph node T cells ($3x10^5$) were cultured with $5x10^4$ stimulatory cells (fresh LC, cultured LC) in round bottom microtiter plates at 37°C, 5% CO_2 for 6 days. One μCi ^{3}H-thymidine was added to each well 16-18 h prior to termination of culture. The cells were harvested with Tomtec Harvester (Wallac, LKB, NJ) and mean (± standard error) radioisotope incorporation of 6 replicated wells were calculated by an automatic β counter (Wallac). BALB/c LC and BALB/c T cells were used to evaluate the syngeneic stimulatory reaction. BALB/c LC and C57BL/6 T cells were used to evaluate the allogeneic stimulatory reaction. In human subject studies, autologous purified peripheral blood T cells and epidermal LC were used. Experiments were repeated at least three times in mice and two times in humans.

Protein purification and analysis. HPLC: Heated serum samples (300 μl) were loaded onto an analytical C-8 column (Phenomenex, Torrance, CA), eluted with a 0 to 70% acetonitrile gradient containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, and collected into several fractions according to the absorption at 215 nm. The fractions were then lyophilized and analyzed by SDS-PAGE. SDS-PAGE: Samples were loaded onto 14% SDS-polyacrylamide gels and run under denaturing condition in the presence of 0.1% SDS and 2.5 mM dithiothreitol. Amino acid sequencing was performed using the pulsed liquid sequencer model 477A (Applied Biosystems, Foster City, CA). The derived sequence was entered using sequence analyzer software Swiss Prot.

USE

In normal skin, the functional properties of Langerhans cells (LC) permit the cells to activate primed T cells, as well as mitogen-sensitive and alloreactive T cells. However,

epidermal LC are incapable of activating naive, antigen-specific T cells *in situ*. It is only when the cells undergo functional transformation, especially in response to GM-CSF, that they acquire the novel capacity to present new antigens to naive, specific T cells. And they do so in draining lymph nodes - not in the skin. It is in respect to these two polar expressions of LC function that our finding of a regulatory role for Hp takes on meaning. Our results indicate that LC resident within the normal epidermis contain Hp, particularly in cytoplasmic vesicles. Moreover, when the cells are removed and placed in tissue culture, they rapidly lose Hp expression - unless exogenous Hp or normal serum is added to the culture fluid. Thus, serum-borne Hp is accessible to epidermal LC under normal circumstances. Moreover, the amount of Hp required to retain LC in their "fresh" functional state is within the range of Hp concentration in cutaneous interstitial fluid. In the aggregate, these facts indicate that LC are normally maintained in their "fresh" functional form within the epidermis by Hp. Epidermal LC functional integrity appears to be dependent upon circulating levels of Hp. Therefore, major shifts in serum Hp levels would have deleterious consequences: on the one hand, excessively high levels would act to prevent induction of immunity to new cutaneous antigens by thwarting functional transformation among LC; on the other hand, excessively low levels of Hp would permit LC to differentiate within the skin, thereby allowing them to activate T cells inappropriately in the cutaneous microenvironment.

The ability of haptoglobin to inhibit functional changes in LC, specifically, to inhibit the ability of antigen presenting cells to activate T cells by interfering with the presentation of immunogenic peptides on class II MHC molecules and by interfering with up-regulation of co-stimulatory molecules, is expected to extend to other dendritic cells, the leukocyte lineage to which LC belong.

Even dendritic cells from peripheral organs have been reported to undergo a functional transformation *in vitro* when cultured in the presence of GM-CSF. After this transformation, the cells display potent co-stimulatory features similar to those described for cultured LC (Girolomoni et al., J. Immunol. 145:2820, 1990; Dai et al., Regional Immunology 5:269, 1993).

Our discovery of this regulatory role for the alpha-1 chain of haptoglobin in LC functional transformation leads immediately to a number of potential products and methods of their use. The alpha-1 chain of haptoglobin or the active portion thereof (which can be determined by conventional methods using fragments of the haptoglobin alpha-1 chain in the assay systems described herein) can be provided as an isolated polypeptide (or stabilized as part of a fusion protein or complexed with the beta chain as the haptoglobin molecule) and used directly in a therapeutic composition to inhibit Langerhans cells and other dendritic cell functional properties and, therefore, can be used to interfere with Langerhans cells/dendritic cell-dependent activation of T cells.

There are many human disorders where inappropriate activation of T cells is a major pathogenic parameter. For example, specific treatable inflammatory skin diseases include psoriasis, mycosis fungoidosis, atopic dermatitis, exfoliative dermatitis and Sezary's Syndrome.

Beyond the field of dermatology, there are an even larger number of autoimmune, T cell dependent inflammations, and infectious diseases that would be treatable by the methods of the invention. Specific examples include prevention of transplantation rejection (e.g., Graft Versus Host Disease), vaccine development, and therapy for infectious diseases where antigen presenting cells play a central role in the pathogenesis. Additionally, the methods of the invention could be used to inhibit Langerhans cell susceptibility to infection with viruses, especially HIV.

In other uses, peptide inhibitors (i.e., antagonists) that bind to the haptoglobin receptor or to the haptoglobin alpha-1 chain and inhibit receptor triggering, or anti-haptoglobin alpha-1 chain antibodies, can be isolated and used to promote antigen presenting cell function, thereby enhancing T cell-dependent immune responses. This treatment would be most effective in circumstances in which antigen presenting cells, at the T cell activation site, were inappropriately flooded with haptoglobin. In addition, the polymorphism of the haptoglobin alpha chain locus would be a useful indicator to diagnose individuals potentially susceptible to diseases resulting from excessive antigen presenting cell stimulation of T cells.

Therapeutic compositions containing human haptoglobin, its alpha-1 chain (or the active portion thereof) or inhibitors of haptoglobin receptor binding may be administered, e.g., by injection (include subcutaneous, intravenous and intramuscular); by oral and inhalation or aerosol therapy; by application to the skin in a lotion, cream, ointment or paste; or by rectal or vaginal suppositories (more suitable for the prevention of AIDS). The active compound can be administered in a dosage of, *e.g.*, about 2 mg/kg/day to 10 mg/kg/day. Optional dosage and modes of administration can readily be determined by conventional protocols. Therapeutic compositions in the methods of the invention may be administered independently or co-administered with another immunosuppressive agent, e.g., cyclosporin-A or corticosteroids.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

CLAIMS

What is claimed is:

1. A method of inhibiting the ability of antigen presenting cells to activate T cells comprising contacting a population of dendritic cells with human haptoglobin, with a haptoglobin alpha-1 chain, or with an active portion of a haptoglobin alpha-1 chain.

2. The method of claim 1, wherein said dendritic cells comprise Langerhans cells.

3. The method of claim 1, wherein said population of dendritic cells is contacted with human haptoglobin.

4. The method of claim 1, wherein said population of dendritic cells is contacted with a haptoglobin alpha-1 chain.

5. The method of claim 1, wherein said population of dendritic cells is contacted with an active portion of a haptoglobin alpha-1 chain.

6. A pharmaceutical composition comprising human haptoglobin, a haptoglobin alpha-1 chain or an active portion of a haptoglobin alpha-1 chain in a pharmaceutically acceptable carrier substance.

7. The pharmaceutical composition of claim 6, wherein said composition comprises human haptoglobin.

8. The pharmaceutical composition of claim 6, wherein said composition comprises a haptoglobin alpha-1 chain.

9. The pharmaceutical composition of claim 6, wherein said composition comprises an active portion of a haptoglobin alpha-1 chain.

10. A method of suppressing T-cell dependent inflammation in a patient comprising the step of administering to said patient a composition according to claim 6.

11. A method of stimulating the ability of antigen presenting cells to activate T cells comprising contacting a population of dendritic cells with an antagonist to human haptoglobin-haptoglobin receptor binding.

12. The method of claim 11, wherein said antagonist is a peptide inhibitor of said human haptoglobin-haptoglobin receptor binding.

13. The method of claim 11, wherein said antagonist is an anti-haptoglobin alpha-1 chain antibody.

14. A pharmaceutical composition comprising an antagonist to human haptoglobin-haptoglobin receptor binding in a pharmaceutically acceptable carrier substance.

15. A method of stimulating a T-cell dependent immune response in a patient comprising the step of administering to said patient a composition according to claim 14.

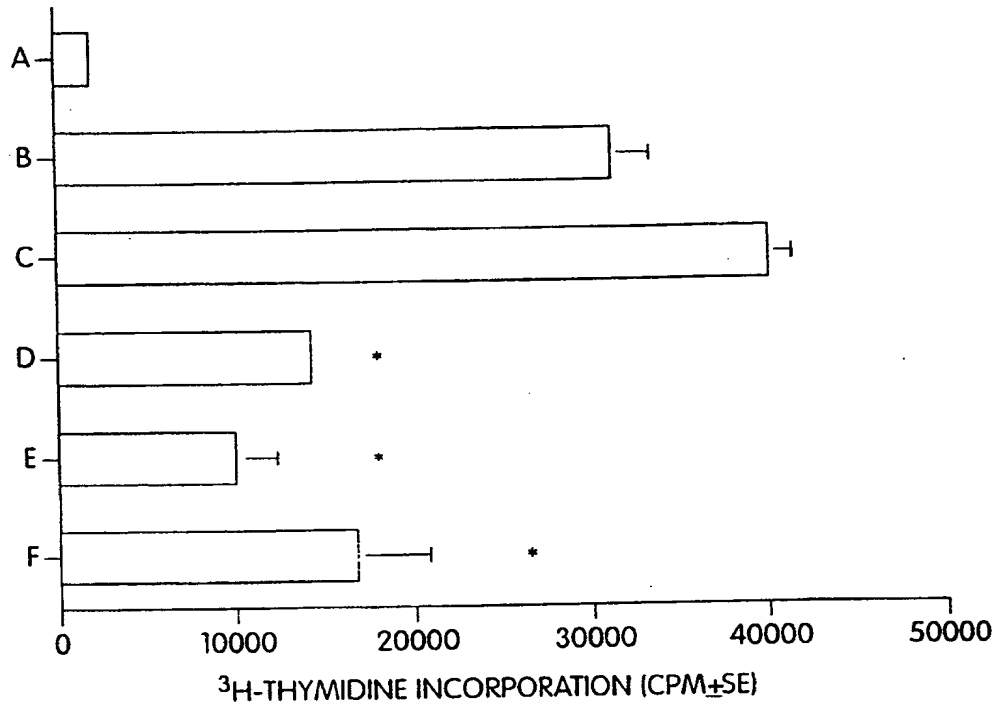
A
B
C
D
E
F
*
*
*
0
10000
20000
30000
40000
50000
^{3}H-THYMIDINE INCORPORATION (CPM±SE)

Fig. 1

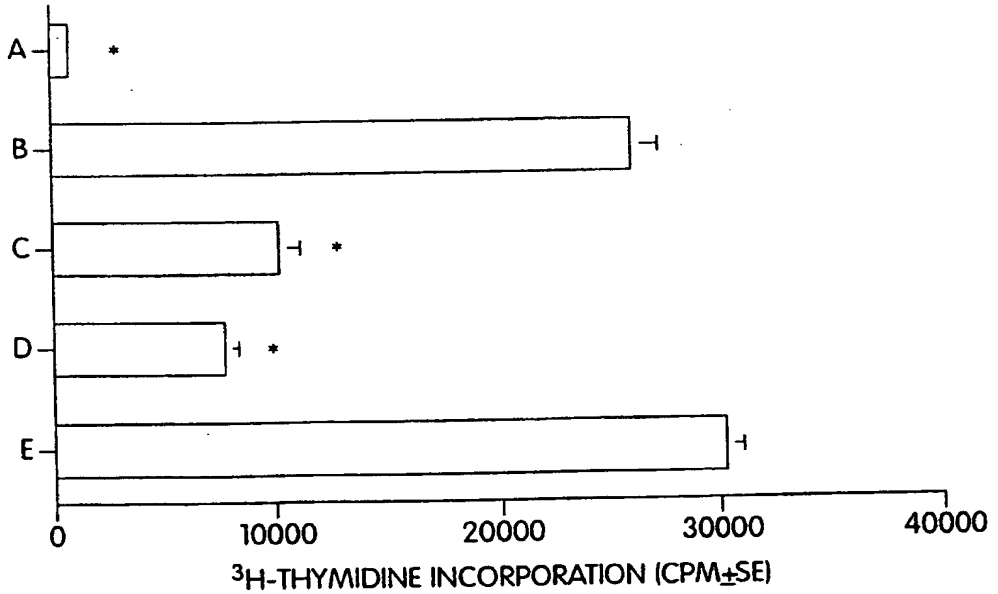
A
B
C
D
E
*
*
*
0
10000
20000
30000
40000
^{3}H-THYMIDINE INCORPORATION (CPM±SE)

Fig. 2A

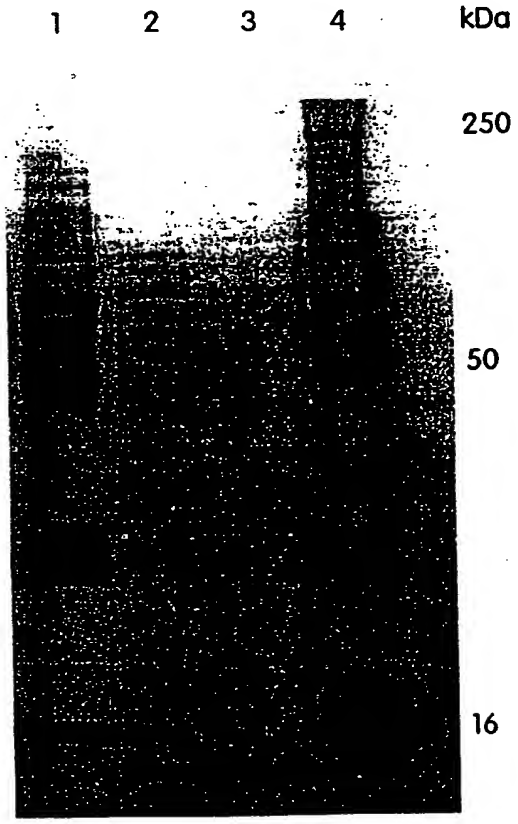
1
2
3
4
kDa
250
50
16

Fig. 2B

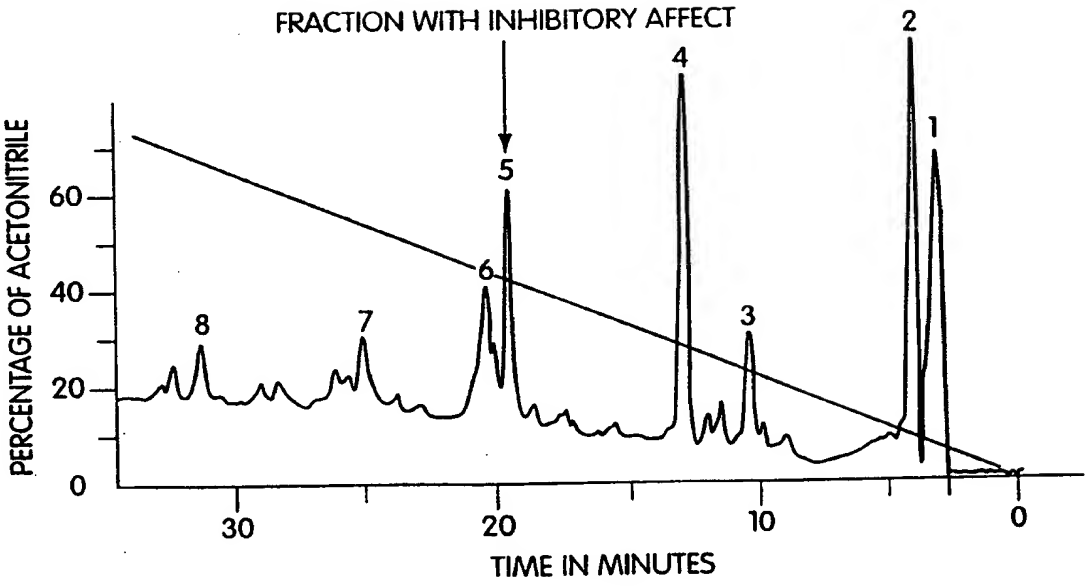
FRACTION WITH INHIBITORY AFFECT
PERCENTAGE OF ACETONITRILE
60
40
20
0
30
20
10
0
TIME IN MINUTES
1
2
3
4
5
6
7
8

Fig. 2C

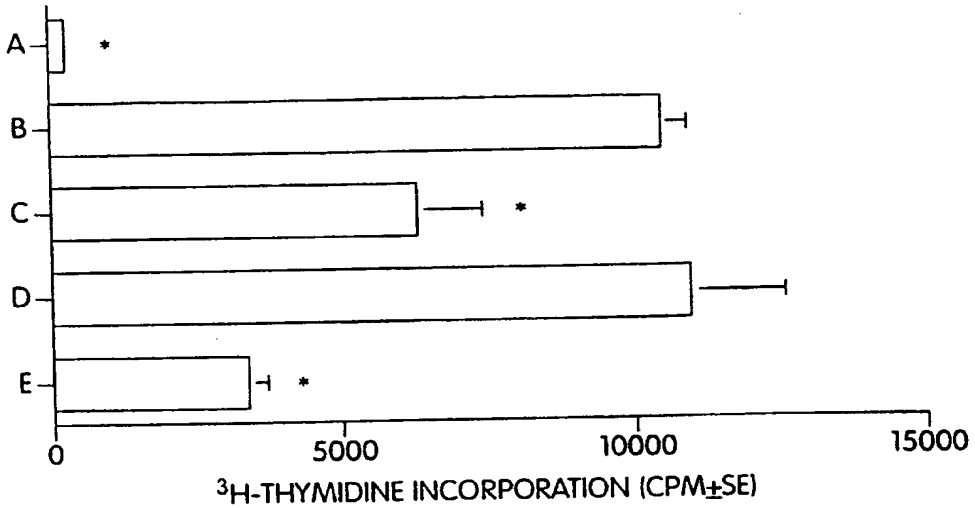
A
B
C
D
E
*
*
*
0
5000
10000
15000
^{3}H-THYMIDINE INCORPORATION (CPM±SE)

Fig. 2D

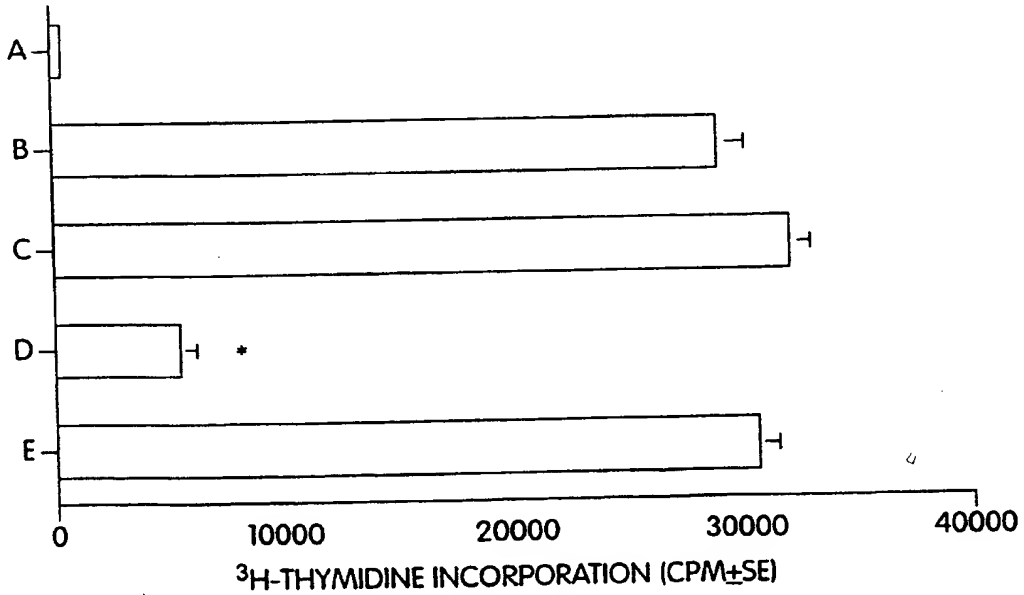
A
B
C
D
E
*
0
10000
20000
30000
40000
^{3}H-THYMIDINE INCORPORATION (CPM±SE)

Fig. 3

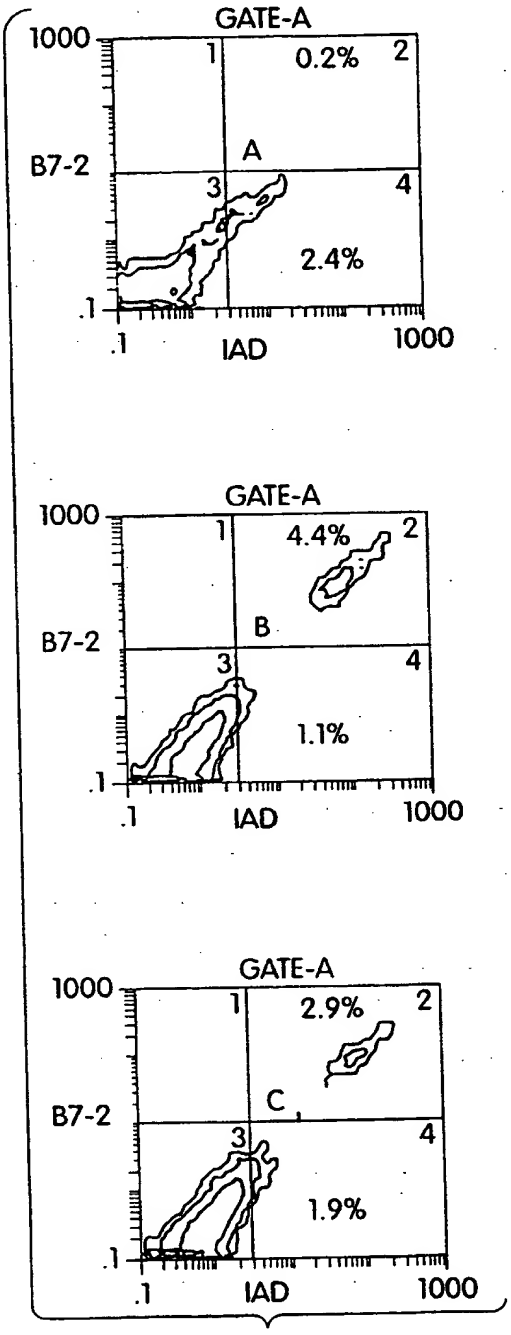
GATE-A
1000
1
0.2%
2
B7-2
A
3
4
2.4%
.1
.1
IAD
1000
GATE-A
1000
1
4.4%
2
B7-2
B
3
4
1.1%
.1
.1
IAD
1000
GATE-A
1000
1
2.9%
2
B7-2
C
3
4
1.9%
.1
.1
IAD
1000

FIG. 4

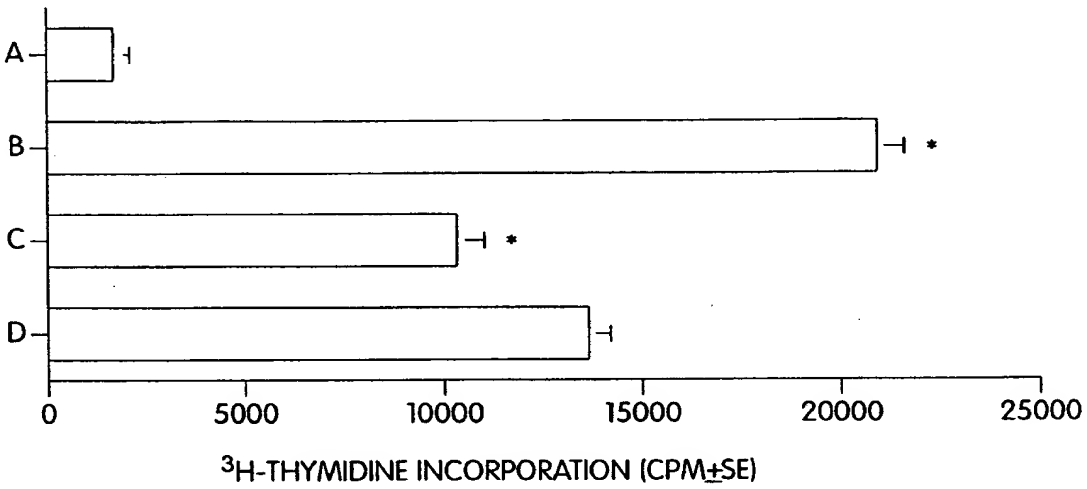
A
B
C
D
*
*
0
5000
10000
15000
20000
25000
3H-THYMIDINE INCORPORATION (CPM±SE)

Fig. 5

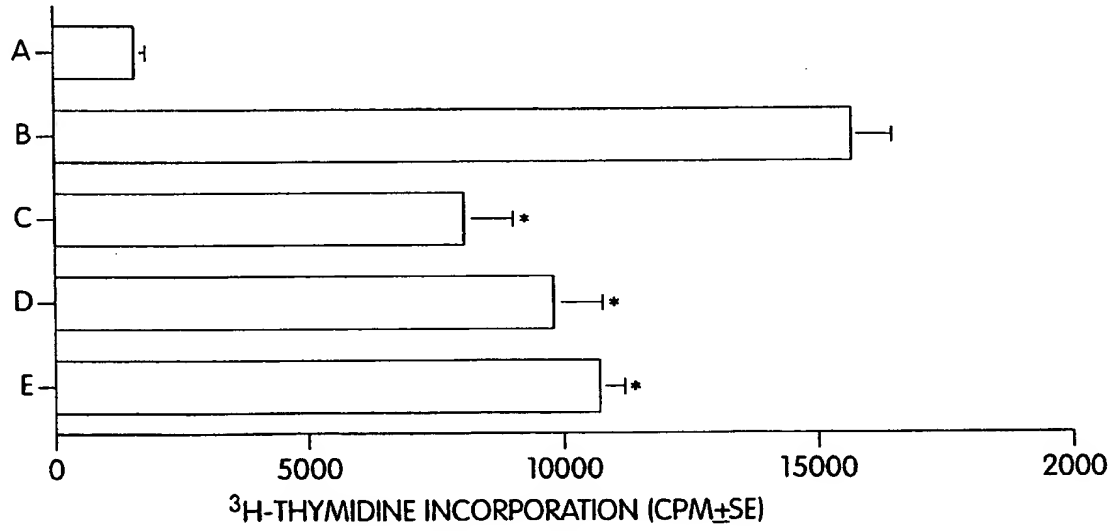
A
B
C
D
E
*
*
*
0
5000
10000
15000
2000
3H-THYMIDINE INCORPORATION (CPM±SE)

Fig. 6

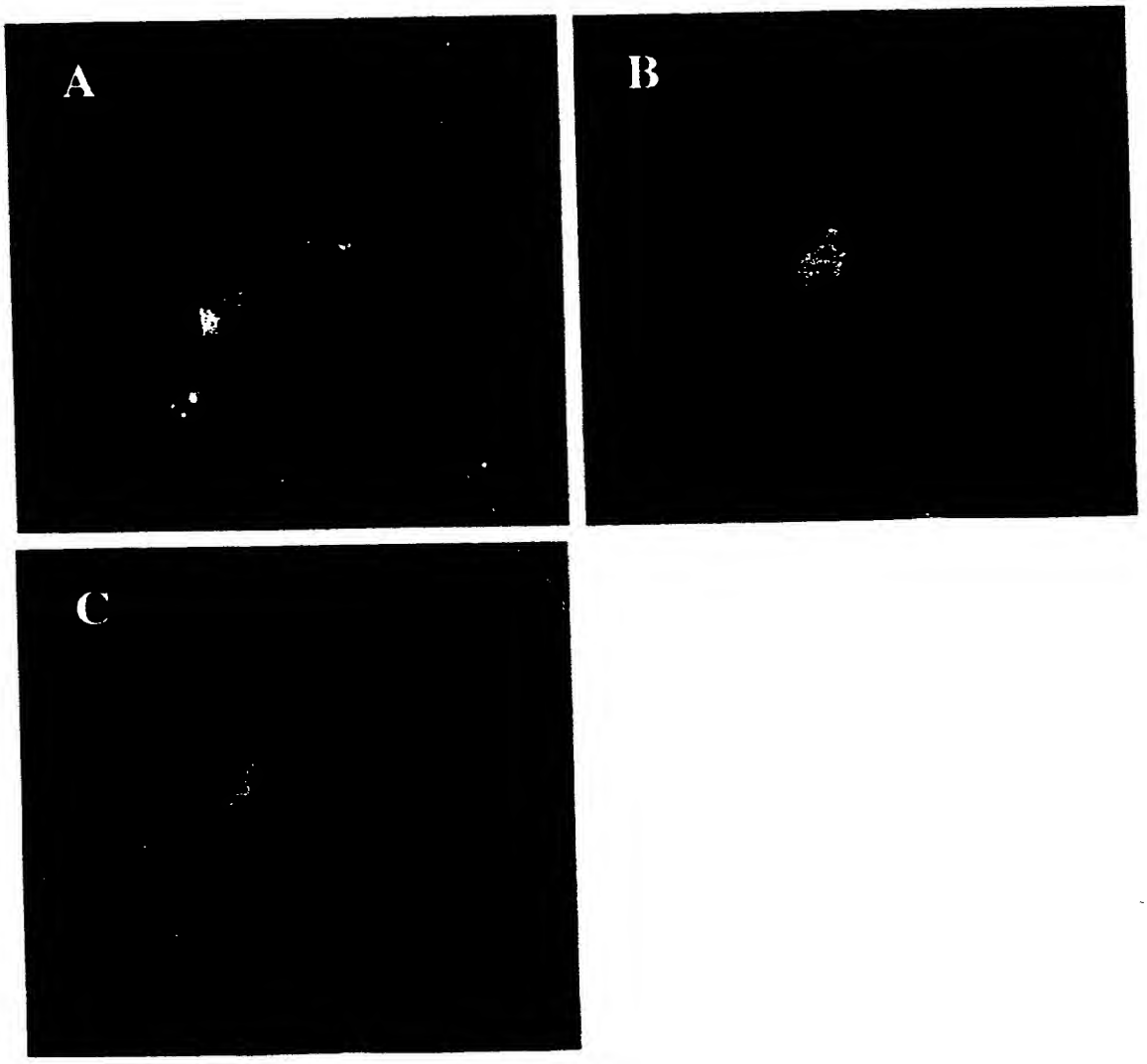

Fig. 7

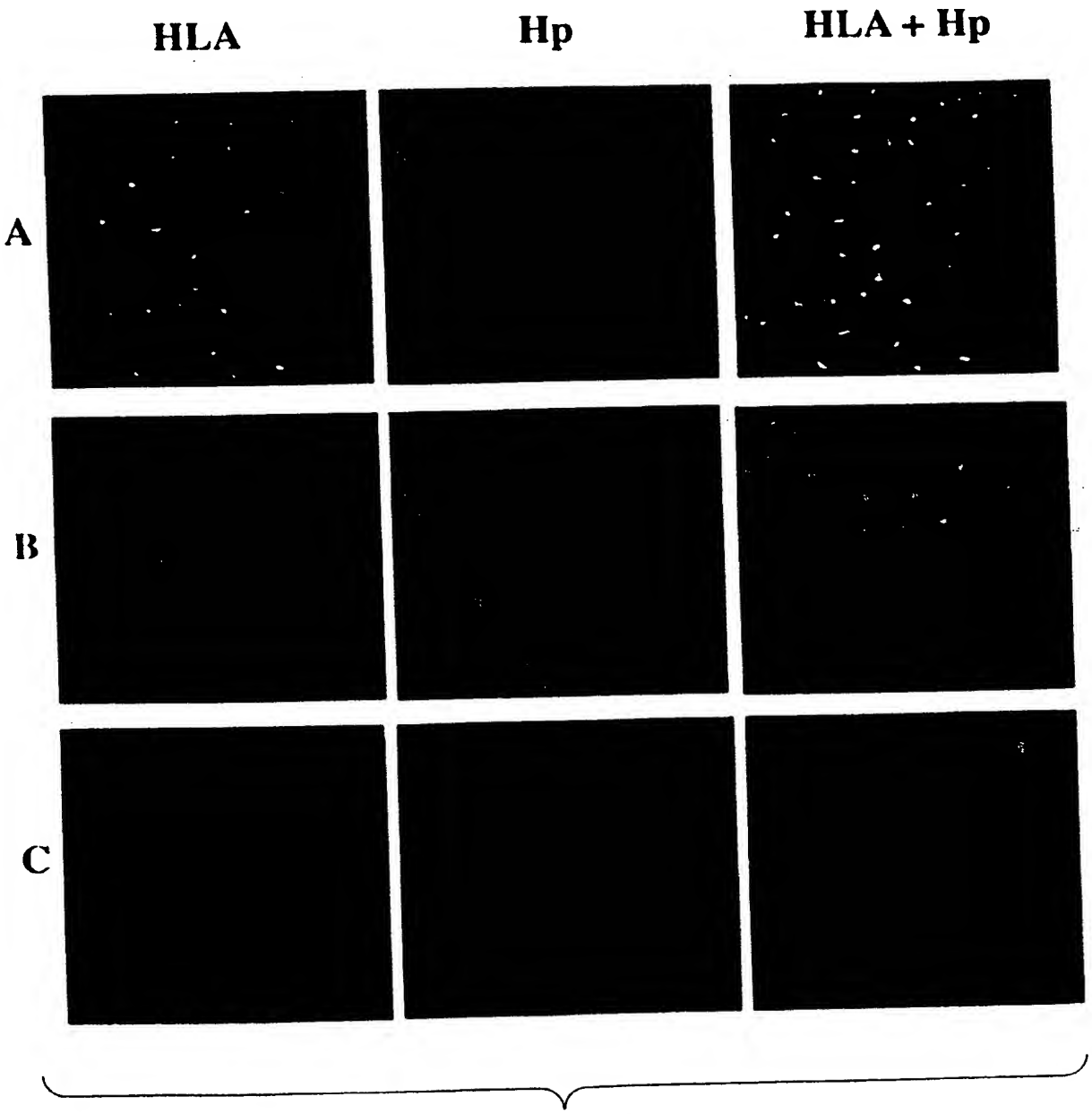

Fig. 8